THE ROLE OF CYTOKINES IN THE GENERATION OF INFLAMMATION AND TISSUE DAMAGE IN EXPERIMENTAL GRAM-POSITIVE MENINGITIS

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The mortality rate of bacterial meningitis remains high (1), and >50% of patients who survive are left with significant neurological sequelae (2). A poor outcome is particularly characteristic of pneumococcal disease, for which the current mortality rate approaches 30% despite effective antibiotic-induced bacterial killing. Pathological changes associated with meningeal inflammation include arteritis, thrombophlebitis, increased intracranial pressure, brain edema, and altered cerebral blood flow (3, 4). Since the amount of inflammation in the subarachnoid space correlates with outcome of disease (5), it is important to understand details of the inflammatory cascade triggered during meningitis in order to design improved therapy. The subarachnoid space is sequestered behind the blood brain barrier and has few, if any, resident host defenses. Thus, the generation of inflammation at this site necessitates communication across the blood brain barrier and recruitment of host defenses from blood to the cerebrospinal fluid (CSF)[1] space. We sought to define the role of cytokines in generating inflammation and tissue damage in the subarachnoid space.

Cytokines are thought to mediate many host responses to bacterial infection. TNF/cachectin and IL-1 play a central role in many responses to infection (6-9), and antibodies to these cytokines can dramatically improve the course of inflammatory events in bacterial sepsis (10). However, since the subarachnoid space is essentially an immunocompromised site, the activities of cytokines in peripheral infections cannot be extrapolated to meningitis. Increased circulating cytokine concentrations have been found inconsistently in states of sepsis (11). Both TNF and IL-1 have been detected in CSF of some patients with meningitis (12, 13), and during Gram-negative meningitis in a rabbit model (14, 15). The sources of IL-1 and TNF in meningitis are unknown. Microglial cells in the brain have been shown to produce IL-1 and

[1] Abbreviations used in this paper: CSF, cerebrospinal fluid; MCP-1, monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein; NAF, neutrophil activating factor; rh, recombinant human.

TNF (16), and it is possible that cerebral capillary endothelia may produce such mediators as has been shown for peripheral vascular endothelium (17).

IL-1 mediates several systemic changes associated with infection, such as fever, neutrophilia, increased hepatic acute phase protein synthesis, hypoferremia, and elevated corticosteroid levels (7, 18). IL-1 exerts biological effects on circulating granulocytes (19) and endothelium (20). IL-1 β has been shown to increase vascular permeability in the rabbit lung (21) and to disrupt the blood brain barrier in the rat (22).

TNF is a cytokine produced by monocytes and macrophages in response to bacterial LPS, IL-2, and mitogens (8). The effects of TNF in vivo include induction of fever (8), induction of IL-1 production by vascular endothelium, and activation of multiple leukocyte functions (6, 10). The histopathologic damage seen in lungs, intestine, kidneys, pancreas, and adrenals in TNF-treated animals resembles that induced by endotoxemia (6).

Wolpe et al. (23, 24) recently characterized two novel cytokines termed "macrophage inflammatory protein" (MIP)-1 and -2, both of which exhibit a strong affinity for heparin. MIP-1 is produced by endotoxin-stimulated macrophages. It is mildly chemotactic for human neutrophils in vitro and induces them to undergo an oxidative burst. When injected subcutaneously into the foot pads of C3H/HEJ mice (endotoxin resistant), a cellular infiltrate comprised primarily of neutrophils is induced. In addition, MIP-1 is an endogenous pyrogen that acts in a prostaglandin-independent manner (25). The other major heparin-binding protein secreted by endotoxin-stimulated macrophages is MIP-2. Sequence analysis indicates that MIP-2 is a member of the platelet factor 4 family. This mediator is an extremely potent chemotactic agent for human neutrophils but induces little chemokinetic activity in vitro. It does not induce an oxidative burst in parallel assays in which MIP-1 is active. MIP-2 also causes a localized inflammatory reaction when injected into foot pads of mice.

The major pathological feature of acute bacterial meningitis is the presence of an inflammatory exudate within the subarachnoid space. We explored the role of TNF, IL-1α, IL-1β, MIP-1, and MIP-2 in generating this inflammatory response. Using an experimental model of pneumococcal meningitis in rabbits, the spectrum of intracranial abnormalities induced by intracisternal administration of each cytokine was found to be different. Specific antibodies to these mediators reduced inflammation and tissue damage in pneumococcal meningitis.

Materials and Methods

Bacterial Components, Cytokines, and Antibodies. Unencapsulated *Streptococcus pneumoniae* strain R6 was grown to mid-logarithmic phase, boiled for 20 min in growth medium, washed, and resuspended in pyrogen-free saline (26). 10^8 cell equivalents were administered intracisternally in a volume of 0.2 ml, and a highly reproducible inflammatory response indistinguishable from natural disease was generated in CSF over 5 h.

The inflammatory response produced by intracisternal instillation of the following cytokines was compared with that of *S. pneumoniae*: recombinant human (rh)TNF-α (2 $\times$ 10^8 U/mg) (Chiron Corp., Emeryville, CA); rhIL-1α (10^7 U/mg) (Lot SM46; courtesy of Dr. P. Lomedico, Hoffman-La Roche, Inc., Nutley, NJ); rhIL-1β (10^6 U/mg) (courtesy of Dr. J. Schmidt; Merck, Sharpe & Dohme Research Laboratories, Rahway, NJ); murine MIP-1 (400 μg protein/ml) (23); and murine MIP-2 (100 μg protein/ml) (24). Recombinant cytokines contained <10 pg endotoxin/ml. The endotoxin content of native cytokines was unknown; to neutralize the possible effects of residual endotoxin in all cytokine preparations, all mediators were tested with and without addition of polymyxin B (50 μg/kg, intracisternally) (Sigma

Chemical Co., St. Louis, MO). All cytokines were >99% pure as assessed by gel electrophoresis.

The ability of the following antibodies to neutralize CSF inflammation was tested. Anti-rh TNF-α (raised in goat and crossreactive with rabbits [27]) and preimmune goat antiserum were a gift of Dr. R. Ulevich (Scripps Clinic, La Jolla, CA). Two goat antibodies were obtained from Dr. Richard Chizzonite (Hoffman-La Roche, Inc.): anti-rhIL-1α and anti-rhIL-1β (1.3 mg antibody/ml; batches 162 and 168, respectively). Antisera to MIP-1 and -2 were produced in rabbits against murine material by one of us (B. Sherry). Antibodies (200 μl/rabbit) were administered intracisternally simultaneously with the cytokines. Control rabbits received antibody alone.

Rabbit Model of Meningitis. The rabbit model was performed according to an established protocol (25, 28, 29). For all experiments, rabbits were challenged in groups of at least four per cytokine per dose. In brief, 2-kg female, specific pathogen-free, New Zealand white rabbits (Hare Marland, Nutley, NJ) were anesthetized with ketamine and xylazine, and a dental acrylic helmet was affixed to the calvarium. 24 h later, the animals were anesthetized with urethane and phenobarbital and placed in a stereotaxic frame. A spinal needle was introduced into the cisterna magna and 300 μl CSF was withdrawn. Heat-killed *S. pneumoniae* R6 (2 × 10⁷/rabbit) or cytokines (TNF, IL-1, MIP-1, MIP-2) were instilled into the subarachnoid space in a volume of ≤200 μl pyrogen-free saline. In some experiments, antibodies or polymyxin B were mixed with the cytokines and administered simultaneously. CSF was withdrawn at 2-h intervals over 6 h and tested for leukocyte density using a counter (Coulter Electronics Inc., Hialeah, FL) and differential cytology using DiffQuik stain (American Scientific Products, Edison, NJ). The CSF samples were centrifuged at 10,000 g for 5 min, and the supernatant fluid was frozen at −70°C until assayed for protein with the BCA method (Pierce Chemical Co., Rockford, IL). After 6 h, animals were killed with an overdose of phenobarbital, and brains were removed immediately for quantitation of brain edema by comparing wet/dry weight (g H_2O/100 g dry weight) (30).

To determine if the pathological changes induced by cytokines required participation of leukocytes, challenge with the cytokines was tested in rabbits treated with intravenous mAb IB4 (1 mg/kg) (obtained from Dr. S. D. Wright, The Rockefeller University) (31, 32). This antibody binds to the CD18 complex of receptors on leukocytes, thereby preventing adhesion of leukocytes to endothelia, a prerequisite to diapedesis (31). The antibody effectively blocks emigration of leukocytes into the CSF in this model (32).

Statistical Methods. Values are presented as the means of four or more rabbits/group ± SD. Comparison between means of groups of rabbits was carried out using the student's t test.

Results

Inflammatory Activity of Cytokines. When instilled into the subarachnoid space, four of five cytokines induced movement of leukocytes across the blood brain barrier into the CSF. Simultaneous administration of polymyxin B with the cytokines had no effect on the magnitude of leukocytosis for any mediator (data not shown). IL-1β had no chemotactic activity, even at a dose of 300 ng/animal, a value 100-fold higher than the lowest active dose of IL-1α. Fig. 1 shows the dose-response curves for chemotactic activity of four mediators: TNF, IL-1α, MIP-1, and MIP-2. IL-1α had the highest specific activity on a per weight basis. However, the onset of leukocytosis was most rapid for TNF (1 h) followed by IL-1α (2 h), then MIP-1 (4 h), and MIP-2 (4 h). MIP-1 was 100 times more potent than MIP-2. The polymorphonuclear leukocyte was the initial cell type present in all exudates (Table I), a feature also characteristic of natural infection with the pneumococcus. However, in the cases of TNF and MIP-1, the predominant cell type shifted to mononuclear cells between 4 and 6 h after challenge (Table I).

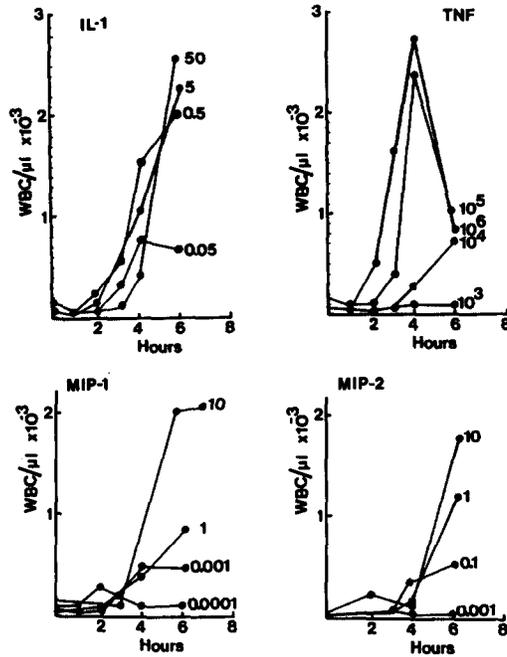When cytokines were mixed with homologous specific antibody and then ad-

FIGURE 1.   Dose-response curves for CSF leukocytosis induced by four cytokines injected intracisternally into rabbits. Concentrations for IL-1α and TNF are indicated in U injected/ animal (0.05 U IL-1α = 1 pg; 10^4 U TNF = 100 ng). Concentrations for MIP-1 and MIP-2 are in μg injected/animal.

TABLE I

Induction of Leukocytosis and Brain Edema by
Intracisternal Instillation of Cytokines

| Cytokine*/antibody (A) | CSF leukocytosis | | Brain edema (H_2O/100 g dry weight at 6 h) |
	Density	Cell type‡	
	cell/μl ± SD		g ± SD
Control§	60 ± 35	–	397 ± 2
Pooled A	35 ± 19	–	398 ± 3
IL-1α	2,343 ± 183	P	407 ± 5
IL-1α + A to IL-1α	612 ± 144‖	P	393 ± 6‖
IL-1β	35 ± 23	–	399 ± 3
IL-1β + A to IL-1β	58 ± 13	–	398 ± 4
TNF-α	1,860 ± 907	P→M	419 ± 3
TNF-α + A to TNF	46 ± 29‖	–	392 ± 5‖
MIP-1	2,308 ± 407	P→M	412 ± 4
MIP-1 + A to MIP-1	346 ± 52‖	–	386 ± 7‖
MIP-2	2,154 ± 168	P	410 ± 6
MIP-2 + A to MIP-2	232 ± 165‖	–	393 ± 1‖

* Minimum dose at which leukocytosis developed in CSF as per Fig. 1 (ng injected: IL-1α, 0.002; IL-1β, 3; TNF, 50; MIP-1, I; MIP-2, 100).
‡ P, polymorphonuclear leukocyte; M, mononuclear leukocyte.
§ PBS diluent for all cytokine preparations.
‖ p < 0.01, compared with cytokine alone.

ministered intracisternally, leukocytosis was inhibited in all cases (Table I). This fact, taken together with the greatly differing purification procedures of MIP-1 and MIP-2, suggests that the low bioactivity of MIP-2 was not due to contamination by MIP-1. The neutralizing activities of heterologous anticytokine antibodies were also tested. The chemotactic effect of IL-1α was not greatly influenced by antibodies to other cytokines (CSF leukocyte densities remained ≥70% of values for IL-1α alone). In contrast, antibody to IL-1α or -β decreased leukocytosis due to TNF by >80%, and anti-TNF reduced leukocytosis caused by MIP-1 or MIP-2 by >75%. The onset of leukocytosis after challenge with IL-1 or TNF was delayed 2 h in animals receiving anti-MIP-1 and anti-MIP-2.

The Ability of Cytokines to Induce Blood Brain Barrier Injury and Brain Edema. Two parameters of brain injury were assessed in cytokine-challenged animals: the appearance of serum proteins in CSF (the hallmark of blood brain barrier breakdown) and the development of brain edema. All cytokines caused an increase in protein concentration in CSF. At 6 h post-challenge, values for each mediator administered as in Table I were as follows (mg/100 ml): control, 50 ± 10; IL-1α, 170 ± 24; TNF, 117 ± 7; MIP-1, 157 ± 22; MIP-2, 170 ± 36. The time course of the increase correlated with the appearance of leukocytes (as shown in Fig. 1). All cytokines also caused a dramatic increase in brain water content (Table I). Homologous antibodies to each mediator decreased the protein influx by a maximum of only 48% (data not shown) but greatly reduced brain edema (Table I).

In rabbits treated with anti-CD18 antibody IB4 (neutrophil adherence dysfunctional), leukocytes failed to accumulate in CSF in response to all cytokines (maximum 266 ± 23 cells/μl using protocol of Table I). Under these conditions, the cytokines also lost the ability to induce brain edema (all brain weights, <399 ± 3.7), but some protein still accumulated in CSF (values for the four cytokines alone were all >130 ± 23 mg protein/100 ml CSF at 6 h post-challenge; cytokine intracisternally plus IB4 intravenously 90–112; IB4 intravenously alone, 81 ± 7).

Neutralization of CSF Inflammation Induced by the Pneumococcus by Anticytokine Antibodies. Anticytokine antibodies were administered intracisternally simultaneously with pneumococci, and the course of inflammation was followed. The onset of leu-

TABLE II

Effect of Anticytokine Antibodies on CSF Inflammation Induced by Pneumococci

Challenge inoculum	CSF leukocytosis*				CSF protein concentration (at 6 h)	Brain edema (H_2O/100 g dry weight at 6 h)
	Density	Onset	Peak	Cell type[‡]		
	cells/μl	*h*	*h*		*mg/100 ml*	*g*
Pneumococcus alone	2,975 ± 139	4	>6	P	352 ± 12	413
+ anti-TNF	399 ± 26[§]	4	>6	P→M	184 ± 27.6[§]	388[§]
+ anti-IL-1α + -β	140 ± 0.7[§]	4	4	P	198 ± 17.7[§]	410
+ anti-MIP-1	2,885 ± 92	6	>6	P→M	125 ± 35.4[§]	412
+ anti-MIP-2	2,842 ± 106	6	>6	P	134 ± 19.8[§]	410
+ Preimmune serum	1,863 ± 142	4	>6	P	327 ± 46	412

* Values are expressed as ± SD.
[‡] As per Table 1.
[§] $p < 0.01$.

kocytosis using pneumococci alone occurred reproducibly at 4 h and the peak value of leukocyte density occurred at 6 h, ranging from 3,500 to 4,000 cells/μl. After treatment with antibodies to TNF or a combination of anti-IL-1α and -β CSF leukocytosis was almost completely inhibited (Table II). In contrast, anti-MIP-1 and anti-MIP-2 did not alter the magnitude of leukocytosis after pneumococcal challenge, but did cause a 2-h delay in the onset of leukocytosis. All antibodies reduced CSF protein concentrations, but only anti-TNF antibody was protective against brain edema.

Discussion

Cytokines are central mediators of inflammatory events in peripheral infections. The studies presented here provide strong evidence that these same mediators also possess a wide range of inflammatory activities in the central nervous system, a site sequestered behind the specialized vascular endothelium comprising the blood brain barrier. However, activity in the periphery did not predict activity in the central nervous system, as exemplified by the inability of IL-1β to cause inflammation in this model. In fact, IL-1α and -β displayed remarkably different inflammatory properties in the central nervous system. Four of the five cytokines (except IL-1β) induced leukocytosis, blood brain barrier permeability, and brain edema. In experimental Gram-negative meningitis in rats or rabbits, peak IL-1 and TNF concentrations in CSF have been measured to be in the range of 5 ng/ml (14, 15). In this rabbit model, only rhIL-1α (not rhTNF or rhIL-1β) induced inflammation at this concentration. Although physiologically relevant concentrations of TNF alone may not induce meningeal inflammation, IL-1 and TNF have been shown to be highly synergistic in peripheral inflammation (33), suggesting TNF may play an important modulatory role in IL-1-induced pathological changes during meningitis. This is also supported by the fact that anti-IL-1α or -β antibodies decreased leukocytosis induced by TNF.

The major chemotoxin during meningeal inflammation remains to be determined. Although chemotactic in this model, IL-1 is not directly chemotactic in vitro. Such direct activity has recently been ascribed to two recently isolated cytokines, neutrophil activating factor (NAF; 34) and monocyte chemoattractant protein 1 (MCP-1; 35). It is possible that the chemotactic activity of IL-1α in this model results from the ability of IL-1α to induce production of either NAF or MCP-1 in vivo. The ability of MIP-2 to induce neutrophil chemotaxis in this model suggests that MIP-2 and the highly homologous protein NAF (34) may have similar biological activities in vivo. Only TNF and MIP-1 were capable of inducing migration of monocytes across the blood brain barrier. This is consistent with the recent finding that MIP-1 is related in peptide sequence to MCP-1, which is monocyte specific (35). The shift in leukocytosis from one cell type to another may relate to the timing of induction of such cell-specific chemoattractant proteins. This is of relevance in vivo, since the appearance of mononuclear cells heralds the resolution of natural pneumococcal meningitis, raising the possibility that this bioactivity might benefit the outcome of disease.

Each cytokine was demonstrated to invoke a unique constellation of pathological findings, underscoring the fact that induction of brain damage in meningitis is the summation of multiple, distinct inflammatory mechanisms. However, determination of the precise roles of and relationships among cytokines in the cascade of inflammation in meningitis awaits purification of all these mediators from one species (i.e.,

the rabbit for this model), since species-specific differences in activities exist. In fact, it has been reported that rabbit IL-1β has some inflammatory activity in this model (36), but less than human IL-1α. Our data would suggest that determination of the activity of rabbit IL-1α will be important.

The hallmark of blood brain barrier injury is the influx of protein from serum into CSF. Such injury was profound in the cases of IL-1α, MIP-1, and MIP-2, but not IL-1β or TNF. IL-1β reportedly induces vascular permeability in the rabbit lung (21) and rat brain (22), suggesting that site- and species-specific effects for this cytokine must be considered. The fact that antibodies to each cytokine did not completely reverse blood brain barrier permeability may indicate that blood brain barrier injury may originate on the vascular side of the blood brain barrier, a site available to the cytokines but not the antibodies, as administered in this model. Alternatively, cytokines have multiple bioactivities that become apparent at different concentrations and that may lack crossreactivity to neutralizing antibody in vivo. All mediators demonstrated the ability to induce brain edema. This effect was lost in animals with neutrophil adhesive dysfunction, indicating that the chemotactic activity of the cytokines rather than a direct effect of cytokines was responsible for generating brain edema.

Antibodies to TNF or a combination of IL-1α and -β greatly decreased meningeal inflammation due to pneumococci. In vitro, pneumococci directly stimulate peripheral blood monocytes to produce IL-1α and -β, but TNF is not produced under the same test circumstances (37). Thus, it is reasonable to suggest that in natural infection, pneumococci may directly stimulate production of IL-1α and -β, which may in turn lead to the production of TNF, which synergistically enhances inflammation. Neutralization of MIP-1 and MIP-2 delayed the onset of leukocytosis in response to pneumococci, suggesting a secondary or modulating role for these cytokines. Thus, in addition to their known effects in the periphery, cytokines are active during natural infection in the central nervous system. These results also define important roles for cytokines in Gram-positive, as well as Gram-negative, infections.

Summary

Cytokines mediate many host responses to bacterial infections. We determined the inflammatory activities of five cytokines in the central nervous system: TNF-α, IL-1α, IL-1β, macrophage inflammatory protein 1 (MIP-1), and macrophage inflammatory protein 2 (MIP-2). Using a rabbit model of meningeal inflammation, each cytokine (except IL-1β) induced enhanced blood brain barrier permeability, leukocytosis in cerebrospinal fluid, and brain edema. Homologous antibodies to each mediator inhibited leukocytosis and brain edema, and moderately decreased blood brain barrier permeability. In rabbits treated with anti-CD-18 antibody to render neutrophils dysfunctional for adhesion, each cytokine studied lost the ability to cause leukocytosis and brain edema. After intracisternal challenge with pneumococci, antibodies to TNF or IL-1 prevented inflammation, while anti-MIP-1 or anti-MIP-2 caused only a 2-h delay in the onset of inflammation. We suggest these cytokines have multiple inflammatory activities in the central nervous system and contribute to tissue damage during pneumococcal meningitis.

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